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**Microbial conversion
of inorganic carbon
to dimethyl sulfide**

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Microbial conversion of inorganic carbon to dimethyl sulfide in anoxic lake sediment (Plußsee, Germany)

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Abstract

In anoxic environments, volatile methylated sulfides including methanethiol (MT) and dimethyl sulfide (DMS) link the pools of inorganic and organic carbon with the sulfur cycle. However, direct formation of methylated sulfides from reduction of dissolved inorganic carbon has previously not been demonstrated. During examination of the hydrogenotrophic microbial activity at different temperatures in the anoxic sediment from Lake Plußsee, DMS formation was detected at 55 °C and was enhanced when bicarbonate was supplemented. Addition of both bicarbonate and H₂ resulted in the strongest stimulation of DMS production, and MT levels declined slightly. Addition of methyl-group donors such as methanol and syringic acid or methyl-group acceptors such as hydrogen sulfide did not enhance further accumulation of DMS and MT. The addition of 2-bromoethanesulfonate inhibited DMS formation and caused a slight MT accumulation. MT and DMS had average $\delta^{13}\text{C}$ values of -55‰ and -62‰, respectively. Labeling with NaH¹³CO₃ showed that incorporation of bicarbonate into DMS occurred through methylation of MT. H₂³⁵S labeling demonstrated a microbially-mediated, but slow, process of hydrogen sulfide methylation that accounted for <10% of the accumulation rates of DMS. Our data suggest: (1) methanogens are involved in DMS formation from bicarbonate, and (2) the major source of the ¹³C-depleted MT is neither bicarbonate nor methoxylated aromatic compounds. Other possibilities for isotopically light MT, such as demethylation of ¹³C-depleted DMS or other organic precursors such as methionine, are discussed. This DMS-forming pathway may be relevant for anoxic environments, such as hydrothermally influenced sediments and fluids and sulfate-methane transition zones in marine sediments.

1 Introduction

Among volatile organic sulfur compounds, the methylated sulfides – dimethyl sulfide (DMS) and, to a lesser extent, methanethiol (MT, or methyl mercaptan) – are the most

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abundant components. The biogeochemical processes involving methylated sulfides in ocean surface waters have received particular attention because of the connection between DMS and climate (Charlson et al., 1987). In anoxic environments, DMS and MT link carbon and sulfur cycles in diverse ways. In contrast to complex organic sulfur compounds formed during early diagenesis that are refractory to biodegradation (Ferdelman et al., 1991), DMS and MT remain reactive and available for microbial processes. Their role as intermediates during remineralization of organic matter has been elaborated in earlier studies and is briefly summarized below and in Fig. 1. Decomposition of S-methyl compounds (compounds with methyl groups bonding to a sulfur atom) such as dimethylsulfoniopropionate (DMSP) and methionine initially yields DMS or MT (Kiene et al., 1990), both of which can be further catabolized by microbes that respire anaerobically. Isolated microorganisms that are known to oxidize methylated sulfides include denitrifying bacteria (Visscher and Taylor, 1993), sulfate reducing bacteria (Tanimoto and Bak, 1994) and methanogens (Lomans et al., 1999a). During degradation of DMS, MT usually accumulates transiently as an intermediate (Lomans et al., 1999b). Whether methylated sulfides can be used to synthesize protein-sulfur during anabolism in anoxic environments is not clear, but evidence exists that pelagic marine bacterioplankton preferentially assimilate methylated sulfides over sulfate or hydrogen sulfide (Kiene et al., 1999).

In addition to processes whereby methylated sulfides are produced from organic sulfur compounds, other processes exist in which methylated sulfides are derived by linking organic or inorganic carbon to $\text{H}_2\text{S}/\text{HS}^-$ (hydrogen sulfide hereafter), either biologically and/or abiotically (Fig. 1). Firstly, O-methyl groups (methyl groups bonding to an oxygen atom) can be transferred microbially to hydrogen sulfide to form MT, with additional methylation under certain circumstances yielding DMS (Lomans et al., 2002). Known O-methyl donors include methanol (van Leerdam et al., 2006) and methoxylated aromatic compounds such as lignin monomers (Lomans et al., 2002). This process is proposed to be the main mechanism contributing to methylated sulfides in DMSP limited freshwater sediments, as evidenced by the strong correlation

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between concentrations of methylated sulfides and hydrogen sulfide in lake sediment (Lomans et al., 1997). Secondly, based on the study of trace methane oxidation of *Methanosarcina acetivorans*, Moran et al. (2007) hypothesized that during anaerobic oxidation of methane (AOM), methane is converted to methylated sulfides, which are subsequently oxidized by sulfate reducers. It is currently not known whether and exactly how such coupling proceeds in AOM organisms. Thirdly, conversion of carbon monoxide (CO) to MT and DMS has also been observed in *M. acetivorans* (Moran et al., 2008), which represents the first finding of microbially mediated incorporation of inorganic carbon into methylated sulfides. The process probably proceeds via stepwise reduction of CO as coenzyme-bound intermediates to form a methyl group and could be coupled to energy conservation via a chemiosmotic mechanism. However, this process has not yet been studied using environmental samples, and the lack of knowledge on CO abundance in aquatic environments hampers evaluation of the significance of this process in natural settings. Lastly, small alkyl-S molecules, except DMS, can be abiotically synthesized from carbon dioxide (CO₂) and hydrogen sulfide in the presence of iron monosulfide under anoxic conditions (Heinen and Lauwers, 1996). This process is accelerated at temperatures higher than 50 °C. The abiotically synthesized alkylated sulfides are considered as building blocks for larger organic molecules in the primitive earth (Huber and Wächtershäuser, 1997). Jointly, these biological and abiotic reactions compete for hydrogen sulfide with other reactions such as the formation of elemental sulfur, polysulfur, metal sulfides and complex organic sulfur compounds.

The finding of formation of methyl sulfides from CO by *M. acetivorans* raises the question whether CO₂ (or bicarbonate at neutral pH), the most abundant dissolved inorganic carbon species in natural waters, can be converted to MT and DMS by microbial activity. Although CO₂ is not a growth substrate of *M. acetivorans*, it is produced in vivo via oxidation of CO and then introduced into the reductive steps by methanofuran (Oelgeschläger and Rother, 2008), a coenzyme shared by autotrophic methanogens. In anoxic environments, reduction of CO₂ to a methyl group is mostly coupled to hydrogenotrophy, such as autotrophic methanogenesis and acetogenesis.

Previous studies of the effect of molecular hydrogen (H₂) on methylated sulfides have yielded inconclusive results. Lomans et al. (1999b) found that after prolonged incubation of anoxic lake sediment under H₂, degradation of methylated sulfides decreased dramatically. In contrast, the addition of H₂ did not affect thiol methylation potential in wetland sediments (Stets et al., 2004).

In an experiment in which we studied hydrogenotrophic reactions as a function of temperature in the anoxic sediment of the eutrophic Lake Plußsee, we detected DMS production after addition of H₂ and bicarbonate at 55 °C. This observation points to the potential presence of a hitherto unrecognized pathway of DMS formation that may be relevant to certain ecosystems functioning at higher temperatures. Our study seeks to establish that this reaction is in fact mediated biologically and to further examine the link among CO₂, H₂, hydrogen sulfide, and methylated sulfides. We performed experiments to characterize the mechanism and to identify the microbial group responsible for the production of methylated sulfides in sediment slurry incubations. The effect of H₂ and bicarbonate on methylated sulfides, methane and acetate was examined, followed by tests aimed at verifying whether the observed DMS formation is related to methyl transfer during O-demethylation from organic substrates. We used a specific inhibitor to investigate the involved enzymatic pathway, which was subsequently supported by a study of the natural carbon isotopic abundance in methylated sulfides. The flow of C- and S-atoms into MT and DMS was further tracked by isotope labeling using ¹³C- and ³⁵S-labeled compounds.

2 Materials and methods

2.1 Study site and sampling

Lake Plußsee (54°10' N, 10°23' E) is a well-studied eutrophic lake located in northern Germany. It has a stable thermal stratification in summer and regularly occurring anoxia in the hypolimnion, leading to high hydrogen sulfide concentrations in the bot-

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tom water (up to $50 \mu\text{mol L}^{-1}$; Eller et al., 2005). Sediment samples used for this study were collected in July 2006 from the deepest part of the lake, i.e., at 28 m using a small multicorer from a rowboat. After sampling, the upper 15-cm portion of triplicate sediment cores was transferred into an air-tight bottle without gas headspace and stored in the dark at 4°C for three months before being used for incubation experiments.

2.2 Slurry preparation

Sediment slurries were prepared anoxically by homogenizing approximately one volume of sediment with one volume of sterilized distilled water. After autoclaving, the distilled water was sparged with nitrogen for at least one hour to remove dissolved oxygen. NaHCO_3 was added as buffer after sparging (final concentration= 1 to 3 mmol L^{-1}) and $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ as reducing agent (final concentration= $50 \mu\text{mol L}^{-1}$). Aliquots of 8 or 10 mL of sediment slurry were dispensed into 16 mL Hungate tubes and sealed with butyl rubber stoppers. The tubes were evacuated three times and flushed with nitrogen or H_2 , and were finally pressurized to 200 kPa. All the tubes were incubated at 55°C in the dark without shaking.

2.3 Substrate and inhibitor amendments

Substrate and inhibitor concentrations are reported for a liquid phase that is ca. 95% (w/w) of the sediment slurry. NaHCO_3 was added to 10 mmol L^{-1} from a CO_2 -stabilized stock solution. Other tested substrates (reported in final concentrations) include: $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, $200 \mu\text{mol L}^{-1}$; MT, $50 \mu\text{mol L}^{-1}$; methanol, $800 \mu\text{mol L}^{-1}$; syringic acid, $100 \mu\text{mol L}^{-1}$. 2-Bromoethanesulfonic acid (BES, 20 mmol L^{-1}) was applied to inhibit methanogenesis.

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2.4 Stable isotope labeling

$\text{NaH}^{13}\text{CO}_3$ was spiked in the middle of an experiment, which was initiated by addition of both H_2 and $10 \text{ mmol L}^{-1} \text{ NaHCO}_3$ at time zero. The amount of added $\text{NaH}^{13}\text{CO}_3$ was determined to set a final ^{13}C label concentration of less than 5% of the background level. To improve the transfer of DMS and MT vapors into the headspace, tubes for carbon isotope determination were stored at -20°C and heated to 60°C for 20 min prior to analysis.

2.5 Radioisotope labeling

In the labeling experiment with H_2^{35}S , $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ was not added to the sterilized and purged distilled water in order not to decrease the specific activity of dissolved inorganic sulfide. Instead, the distilled water was added with resazurin (final concentration = 1 mg L^{-1}) and reduced slowly with freshly-prepared sodium dithionite solution until the liquid became colorless. Aliquots of sediment slurry of 25-mL were poured through a funnel into 120 mL serum vials and amended with H_2 and $10 \text{ mmol L}^{-1} \text{ NaHCO}_3$. Autoclaved slurries (120°C , 25 min) were used as control to account for non-biological reaction and/or processing artifacts.

Radioactive sulfide was produced biologically and purified to remove S-bearing byproducts (T. Holler, unpublished data). The specific activity was $50.9 \text{ MBq mmol}^{-1}$ in the form of Zn^{35}S . Each serum vial was supplemented with H_2^{35}S gas generated by acidifying a defined volume of Zn^{35}S suspension (equivalent to 59.5 kBq). The injected H_2^{35}S should have resulted in a final hydrogen sulfide concentration of $37 \text{ } \mu\text{mol L}^{-1}$ ignoring partitioning into solid S phases.

To allow for equilibration of H_2^{35}S in the serum vials, the time-zero sampling was performed after two hours of incubation. The clear supernatant of the sediment slurry was collected with a plastic syringe fitted with a hypodermic needle, filtered ($0.2 \text{ } \mu\text{m}$), and the filtrate was injected directly into a 5% zinc acetate solution for determination of ra-

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5 dioactivity in the dissolved fraction and concentration of hydrogen sulfide. The samples for determining hydrogen sulfide concentration were stored at -20°C until analysis. The remaining sediment slurry was treated with 20% of zinc acetate and 1 mmol L^{-1} NaOH and left for two hours at 4°C in order to trap H_2S , MT and carbon disulfide vapors (Adewuyi and Carmichael, 1987) into the liquid or solid phase. We interpreted the remaining volatile radioactivity to represent DMS, which was trapped using the method slightly modified from Kiene and Linn (2000). The original butyl stopper on the serum vial was exchanged for a butyl stopper that had an Eppendorf centrifuge vial (1.5 mL) attached to it. Inside the Eppendorf vial was a strip of Gelman AR glass fiber filter treated with freshly prepared 3% H_2O_2 solution. The serum vials were placed in the dark at room temperature and the sediment slurries were stirred for $>6\text{ h}$. The traps were then removed, and the strips placed in 5 mL scintillation vials with scintillation fluid for determination of ^{35}S -radioactivity. Tests with $50\text{ }\mu\text{mol L}^{-1}$ DMS showed that after trapping, DMS decreased to a level below the detection limit of the flame ionization detector. ^{35}S -DMS was not available to determine the exact trapping efficiency, but the trapping efficiency for H_2S in bicarbonate-buffered solution was higher than 90%. The remaining sediment slurries were subjected to a two-step cold distillation (Fossing and Jørgensen, 1989) to investigate the distribution of radioactivity in acid volatile sulfide (AVS: $\text{H}_2\text{S}+\text{FeS}$) and chromium reducible sulfur (CRS: $\text{S}^{\circ}+\text{FeS}_2$). N, N-dimethylformamide was applied in the second step to improve the yield of elemental sulfur (Kallmeyer et al., 2004).

Assuming that only the dissolved fraction could be responsible for formation of methylated sulfides, we calculated the average production rate of DMS derived from doubly methylated hydrogen sulfide ($\text{Rate}_{\Sigma\text{H}_2\text{S}+2\text{Me}}$) during a labeling period using the following equation

$$\text{Rate}_{\Sigma\text{H}_2\text{S}+2\text{Me}} = \frac{a_{\text{trap}}}{a_{\text{dis}}} \times \{\text{H}_2\text{S}\} \times \frac{1}{t}. \quad (1)$$

Here $\text{Rate}_{\Sigma\text{H}_2\text{S}+2\text{Me}}$ is expressed as $\mu\text{mol (L of slurry)}^{-1} \text{ d}^{-1}$. a_{trap} is the radioactivity of the trapped pool, a_{dis} is the activity in the dissolved pool of the sediment slurry,

{H₂S} is the concentration of hydrogen sulfide per volume of sediment slurry (μmol (L of slurry)⁻¹), and *t* is the number of days during which the reaction was run. Since H₂³⁵S is likely to be oxidized into aqueous sulfur oxyanions even under anoxic conditions (Elsgaard and Jørgensen, 1992), *a*_{dis} can be an overestimation of the H₂³⁵S pool, leading to underestimated values of Rate_{ΣH₂S+2Me}.

2.6 Analytical techniques

Concentrations of methane and methylated sulfides were determined by headspace analysis. Care was taken to maintain the incubation temperature during gas sampling. An aliquot of 100 μL gas was taken from a headspace of 6 to 8 mL using a Hamilton gas-tight syringe for on-column injection via a programmable temperature vaporizing inlet. A gas chromatograph (Trace GC Ultra, ThermoFinnigan) equipped with a CP-PoraBOND Q (Varian Inc.) column and a flame ionization detector was used to quantify the compounds. The column temperature was programmed from 60 °C (1 min isothermal) to 240 °C (2 min isothermal) at a rate of 40 °C min⁻¹. The distribution coefficients for DMS and MT at 55 °C are 6.9 and 4.3, respectively (Przyjazny et al., 1983). Calibration was made with standards prepared anoxically from chemicals. The limits of detection (LOD) were 0.4 μmol L⁻¹ for MT and 0.5 μmol L⁻¹ for DMS under the described analytical conditions.

For carbon isotope analysis, the same model of GC was coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer via a Finnigan combustion interface-III. One milliliter of gas was injected into the split/splitless inlet. A column and a temperature program identical to those described above were used. Values of δ¹³C relative to that for Vienna-PeeDee Belemnite are defined by the equation δ¹³C (‰) = (R_{sample}/R_{standard} - 1) × 1000 with R = ¹³C/¹²C and R_{standard} = 0.0112372 ± 2.9 × 10⁻⁶. Internal precision of δ¹³C was better than ±0.1‰ (one standard deviation). We used two types of standards to evaluate the isotopic accuracies of methylated sulfides at low concentrations: (1) gaseous standards were prepared by injecting small amount

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of liquid chemicals into helium-flushed, water-free headspace vials that were allowed to equilibrate at 60 °C. Isotopic values of the gaseous standards were not influenced by partition of methylated sulfides into aqueous solution and were taken as ‘real’ values. (2) Solution standards of different concentrations were prepared under a helium headspace with a defined volume of water. By comparing the isotopic values of the solution standards with those of the gaseous standards, we found that at concentrations lower than 5 $\mu\text{mol L}^{-1}$, there was a positive shift for MT (up to 4.5‰) and a negative shift for DMS (up to 2.5‰). Since a strict relationship between the correction factor and concentration was not established, we did not correct the $\delta^{13}\text{C}$ values of MT, which had concentrations lower than the threshold during the whole course of the incubation experiments.

Radioactivity was determined by liquid scintillation counting (Packard 2500 TR) with a counting window of 4 to 167 keV without luminescence correction. The scintillation cocktail Lumasafe Plus (Lumac BV, Holland) was mixed with the zinc acetate-fixed dissolved fraction and the glass fiber strips. The counting efficiency was higher than 95%. Counting time was 10 min for all samples. The concentration of hydrogen sulfide was determined colorimetrically by the methylene blue method (Cline, 1969).

For acetate analysis, sediment slurries in Hungate tubes were centrifuged at 800×g for 10 min, and 1 mL supernatant was removed with a plastic syringe fitted with a 21-gauge needle and filtered through a 0.45- μm Rotilabo Teflon syringe filter. The filtrates were stored at –20 °C until analysis. Acetate was measured using a high performance liquid chromatograph equipped with a Nucleogel Column (Machery-Nagel Inc.) and a photodiode array detector. Calibration curves were generated using standards prepared gravimetrically from sodium acetate. The detection limit for acetate was 10 to 15 $\mu\text{mol L}^{-1}$.

2.7 Statistical analysis

The LOD of instrumental methods was assessed statistically by the equation $\text{LOD} = Y_B + 3 \times S_{y/x}$, where Y_B represents the signal of instrumental background and was estimated

by the intercept of a calibration curve, and $S_{y/x}$ stands for the deviation of Y values (see Miller and Miller, 2005, for detailed description). The effect of individual treatments was tested using a one- or two-tailed t test with α set at 0.05.

3 Results

3.1 Addition of substrates

In the first experiment, we examined the effects of H_2 and bicarbonate on the dynamics of DMS and MT formation at 55 °C. We also monitored the concentrations of methane and acetate, the major carbon pools whose formation can potentially compete for H_2 and bicarbonate in this system. Compared to the control without any addition (Fig. 2a), there was no stimulation of DMS accumulation when H_2 was added as the single substrate (Fig. 2b). However, addition of bicarbonate resulted in an increase of DMS (Fig. 2c), and simultaneous addition of both H_2 and bicarbonate further enhanced DMS accumulation (Fig. 2d). The average accumulation rate was 2.1 $\mu\text{mol DMS (L of slurry)}^{-1} \text{d}^{-1}$ between days 3 and 9. Subsequent tests with the same treatment yielded a range of maximal accumulation rates from 1.3 to 2.4 $\mu\text{mol DMS (L of slurry)}^{-1} \text{d}^{-1}$. In the presence of H_2 and bicarbonate, DMS concentration in the autoclaved sediment was significantly lower than that in the active sediment. For MT, the final concentration after nine days of incubation was highest in the control (Fig. 2e) and lowest when both H_2 and bicarbonate were added (Fig. 2h), but there was no apparent stoichiometric relationship between the kinetics of DMS and MT accumulation. MT concentration was below LOD in the autoclaved sediments (Fig. 2e, h). Methane accumulation was suppressed by addition of H_2 (Fig. 2j, l) but slightly enhanced by amendment with bicarbonate (Fig. 2k). The acetate concentrations at time zero in the active and autoclaved sediments were both below 10 $\mu\text{mol L}^{-1}$. The effect of H_2 and bicarbonate as single substrates on acetate formation was minor and negligible, respectively (Fig. 2n, o). In combination H_2 and bicarbonate resulted in a 2.7-fold increase of acetate (Fig. 2p).

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Note that the concentration of acetate was much higher than DMS, MT and methane by one to three orders of magnitude at the end of incubation. Although addition of bicarbonate alone already enhanced DMS formation (Fig. 2c), in the following experiments we added bicarbonate together with H₂ in order to obtain higher yields of DMS and to facilitate labeling studies.

To test whether the observed DMS formation is related to the activity of O-demethylation, we examined the effects of sodium sulfide, MT, methanol and syringic acid under either a nitrogen or a H₂ headspace (Table 1). Experimental concentrations of each of these compounds were in the range that generated microcosm response in previous studies of freshwater sediment (Lomans et al., 1997; Stets et al., 2004). The results indicated that the presence of H₂ and bicarbonate was the major factor leading to improved DMS formation; none of the four substrates significantly improved DMS formation after 12 days of incubation. The effects of sodium sulfide, methanol or syringic acid on MT formation were either minor or insignificant. The added MT decreased over time and reached 30% of the initial values after 12 days of incubation. An autoclaved control confirmed that the disappearance of MT was driven by abiological processes (data not shown).

3.2 Inhibition tests

Reduction of bicarbonate with H₂ in anoxic sediment is commonly associated with activities of either acetogens or methanogens. Acetogenesis was very active in our incubated sediment but could not be specifically inhibited to examine the corresponding effects on formation of methylated sulfides. On the other hand, the role of methanogens can be evaluated by BES, an inhibitor for methanogenesis (Chidthaisong and Conrad, 2000). Relative to the positive control (Table 2), DMS formation was 60% inhibited ($P = 0.024$, one-tailed t-test), whereas MT accumulation increased slightly ($P = 0.044$, one-tailed t-test). Since methane production was already suppressed in the presence of high H₂ partial pressure (Fig. 2i–l), BES did not significantly lower the methane level ($P = 0.182$, one-tailed t-test) in this experiment in which 200 kPa H₂ was applied. BES

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had no effect on the formation of acetate.

3.3 Stable carbon isotopic compositions under non-labeling and ^{13}C labeling conditions

The stable carbon isotopic compositions of methylated sulfides provide additional evidence on the enzymatic pathways involved in their formation. Under the non-labeling condition, DMS was ^{13}C -depleted with δ -values as negative as -62‰ on day 6, and increased slightly to -56‰ at the end of incubation (Fig. 3b). The analyzed $\delta^{13}\text{C}$ values of MT were -55‰ and -53‰ on days 6 and 12 (Fig. 3d), respectively, but the actual $\delta^{13}\text{C}$ values of MT should have been more negative, since the MT concentrations were below the threshold value of $5\ \mu\text{mol L}^{-1}$ (cf. Materials and methods). Methane production was low and the $\delta^{13}\text{C}$ values remained around -50‰ during the course of incubation (Fig. 3f).

In parallel, we performed a ^{13}C -labeling experiment with $\text{NaH}^{13}\text{CO}_3$ to investigate the incorporation of bicarbonate into DMS. If bicarbonate was used directly to form one of the methyl groups in DMS rather than exerting an indirect effect that favored DMS formation (e.g., via buffering the aqueous solution), we would expect rapid labeling after $\text{NaH}^{13}\text{CO}_3$ was added. The experimental results support this hypothesis: one day after addition, the $\delta^{13}\text{C}$ values of DMS became enriched by nearly 40‰ and ended at $+119\text{‰}$ (Fig. 3b). MT showed a much slower but significant ($P = 0.002$, one-tailed t-test) enrichment, and reached -24‰ by the end of the experiment. ^{13}C -enrichment of methane was merely 9‰ (Fig. 3f), a finding that agrees with our observation that methanogenesis via reduction of CO_2 was low.

3.4 Labeling with H_2^{35}S

The result of the ^{13}C -labeling experiment indicates incorporation of bicarbonate with MT, but the source of MT remains unclear. The effects of sodium sulfide and syringic acid on MT formation were insignificant (Table 1), and the slight ^{13}C enrichment of

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MT in the labeling experiment is not a conclusive proof for methylation of hydrogen sulfide by reduced bicarbonate. With the radiotracer experiment with H_2^{35}S , we sought to better characterize the reaction of formation of DMS – via the intermediate MT – from hydrogen sulfide. The MT pool targeted in this experiment may derive from the reactions of hydrogen sulfide with inorganic carbon or non-S-methyl compounds such as O-methyl pools (Fig. 1).

At time zero (after two hours of incubation), the dissolved fraction contained less than 10% of total added radioactivity (Fig. 4c, d). A significant amount of H_2^{35}S entered the solid phase AVS (most likely FeS) and CRS fractions via isotope exchange (Fig. 4a, b; Fossing et al., 1992). The trapped radioactivity in non-autoclaved samples was much higher than in the autoclaved control (Fig. 4e, f; $P = 0.008$, one-tailed t-test), suggesting (1) that DMS formed biologically, and (2) that only a minor component of H_2^{35}S vapor was incompletely fixed and trapped. In the non-autoclaved samples, the trapped radioactivity increased most rapidly between days 3 and 7 and finally reached 2.5% of the dissolved radioactivity. We obtained an average $\text{Rate}_{\Sigma\text{H}_2\text{S}+2\text{Me}}$ of $0.06 \mu\text{mol (L of slurry)}^{-1} \text{d}^{-1}$ over the whole period of incubation and a higher rate of $0.1 \mu\text{mol (L of slurry)}^{-1} \text{d}^{-1}$ between days 3 and 7. The rates are in the range of reported sulfide-dependent MT production rates in freshwater sediment at low hydrogen sulfide concentrations (Lomans et al., 1997), but represent <10% of the ambient DMS production rate determined by the kinetics of total DMS formation in this study.

4 Discussion

Our experimental data showed that both DMS and MT were of biogenic origin and depleted in ^{13}C . However, both compounds responded differently to addition of substrates and inhibitors, and exhibited different patterns of ^{13}C incorporation. Therefore, DMS and MT formation will be discussed separately, followed by a note on the implication of these processes for natural anoxic environments.

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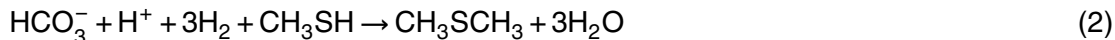
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4.1 Microbial DMS formation

Our observation indicates that dissolved inorganic carbon in anoxic aquatic environments can be incorporated into DMS. The distinct effects of H₂ and bicarbonate in stimulating DMS formation, the slight accumulation of MT when DMS production was inhibited, as well as the more rapid incorporation of inorganic ¹³C into DMS compared to MT, suggest the following reaction:



$\Delta G^{\circ\prime} = -159.8$ kJ per reaction.

The standard-state free energy for the proposed reaction was calculated using thermodynamic data for aqueous species listed in Scholten et al. (2003). The production rate is consistent with the previously observed kinetics in other freshwater environments (Lomans et al., 1997; Stets et al., 2004). Addition of MT did not further stimulate DMS formation (Table 1), suggesting that this process is not limited by MT. Since addition of methanol or syringic acid failed to stimulate DMS production, this process is not related to O-demethylation.

DMS under the non-labeling condition was ¹³C-depleted with δ -values as negative as -62‰ (Fig. 3b). According to Eq. (2), one of the methyl groups was from MT and the other from reduced bicarbonate. Since the measured $\delta^{13}\text{C}$ value of MT in experiments without label addition was around -54‰, a simple mixing model provides a rough estimate of -70‰ for the $\delta^{13}\text{C}$ value of the methyl group from reduced bicarbonate. Compared with the $\delta^{13}\text{C}$ value of the added NaHCO₃ (-2.2‰; data not shown), the strong ¹³C-depletion of the methyl group is consistent with a kinetic isotopic effect typical for H₂/CO₂ metabolizers such as autotrophic methanogens (Whiticar, 1999) or acetogens (Gelwicks et al., 1989).

Suppression of DMS production by BES further suggests that methanogens are involved in DMS formation. Moran et al. (2008) previously discussed the enzymatic steps of *M. acetivorans* to produce methylated sulfides. We hypothesize that the

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5 same pathway is active with CO₂ rather than CO as the direct precursor (Fig. 5). Normally, coenzyme M (CoM-SH) receives the methyl group from N⁵-methyl-H₄MPT:CoM-SH methyltransferase (Mtr) complex to form methyl-CoM, which releases methane by forming heterodisulfide with coenzyme B (CoB-SH) by the catalysis of methyl-CoM reductase (Mcr). Under the condition of DMS production, the methyl group is transferred from methyl-CoM to MT. As Moran et al. (2008) pointed out, such a shortcut to regenerate CoM-SH is feasible, owing to the low energy barrier in the activation step of methanogenic DMS consumption by methylthiol:CoM methyltransferase (Mts) ($\Delta G^{of} = 0.35$ kJ per reaction; Tallant et al., 2001). The overall energy yield for the methanogenic DMS production is theoretically lower than normal methanogenesis, as the H⁺-pumping step of heterodisulfide reduction is bypassed and energy conservation is restricted to the Na⁺-pumping Mtr complex (Hedderich and Whitman, 2006). From an energetic point of view, therefore, DMS might be considered as a by-product of inefficient methanogenesis.

15 DMS formation may also be a response to environmental stress. For instance, Moran et al. (2008) attributed the production of methylated sulfides by *M. acetivorans* to the high CO concentration (300 kPa) in their cultures. CO likely inhibits Mcr, causing CoM-SH to be sequestered and energy production stopped. Transferring the methyl group to hydrogen sulfide or MT can be a shortcut to regenerate CoM-SH while bypassing Mcr. In our incubation, the headspace CO concentrations were lower than 0.5 Pa (data not shown) and were unlikely to have a marked inhibitory effect. Nevertheless, the methane accumulation rates at 55 °C in Lake Plußsee sediment were indeed much lower by one to two orders of magnitude than rates at 27° and 40 °C (data not shown). This suggests that, while the mesophilic community was under high temperature stress, an activation of a thermophilic population of methanogens did not occur. Additionally, it has been repeatedly observed that methanogenesis in the Plußsee sediment was inhibited by high partial pressure of H₂ (Nüsslein and Conrad, 2000; Heuer et al., 2010; Fig. 1). This is probably due to accumulation of certain fermentation products, such as propionate, which could exert an inhibitory effect on certain groups of methanogens,

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especially when pH is decreased (Yeole et al., 1996; Wang et al., 2009). On the other hand, the elevated temperature also improved the availability of substrates such as MT and H₂ via acceleration of organic matter breakdown, making the DMS-forming pathway thermodynamically feasible for methanogens.

Sequences retrieved from fresh Lake Plußsee sediment using the *mcrA*-gene primer showed the greatest homology with members of the *Methanomicrobiales* (Heuer et al., 2010), which are strict H₂/CO₂ metabolizers and are unlikely to possess Mts required for the transfer of methylated sulfides (Fig. 5). Therefore, it is likely that the observed DMS formation was performed by a minor population of methanogens that, like some members of the *Methanosarcinales*, are capable of using both H₂/CO₂ and methylated sulfides (Kendal and Boone, 2006).

4.2 Microbial MT formation

Autoclaved controls confirmed that MT in our system is also of biological origin, and the natural $\delta^{13}\text{C}$ -values are very negative ($< -50\%$). In contrast to some previous studies suggesting that methyl transfer from methoxylated aromatic compounds to hydrogen sulfide is a main source of MT in freshwater sediment (Lomans et al., 1997, 2002), addition of sodium sulfide, methanol and syringic acid to our microcosm failed to stimulate MT formation. By labeling with H₂³⁵S we were able to quantify the inventory of methylated hydrogen sulfide that finally entered the DMS pool. The minor supply of MT derived directly from hydrogen sulfide does not support the accumulation rate of DMS that is supposed to derive from MT (Eq. 2). Taken together, our experiments suggest that the DMS production proceeded via MT derived mostly from organic-S pools. However, we can not yet from these experiments identify the mechanism of MT formation.

Nevertheless, the carbon isotopic composition of MT at natural abundance levels is intriguing and deserves further discussion. The main question is: How can we explain the ¹³C depletion of MT that we observed under the non-labeling condition? Hydrogenotrophic bicarbonate reduction is usually considered the main process that

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generates the ^{13}C -depleted methyl group. If the slight enrichment of MT in the ^{13}C -labeling experiment reflects the signal of bicarbonate incorporation (Fig. 3), the contribution of bicarbonate reduction must be very minor, otherwise the small carbon pool of MT and its inferred rapid turnover should have allowed a pronounced labeling signal.

Alternatively, the slight enrichment of MT can also be explained by demethylation of ^{13}C -labeled DMS. Such a process would also augment the MT pool with ^{13}C -depleted methyl group produced during bicarbonate reduction under the natural (non-labeling) condition. ^{13}C - or ^{35}S -labeled DMS will be required to confirm the presence of DMS demethylation experimentally. A second possible source of a ^{13}C -depleted methyl group would be methoxylated aromatic compounds: Keppler et al. (2004) reported that the methyl pool in lignin has $\delta^{13}\text{C}$ values as negative as -66% . This possibility is nevertheless not supported by our results from the substrate tests and H_2^{35}S -labeling experiment. A last potential source that has received little attention but cannot be ruled out is the S-methyl pool of amino acids. MT accumulated rapidly after addition of methionine in the Plußsee sediment (data not shown), but we have no information on the pool size of free methionine and its endogenesis from enzymatic hydrolysis of macromolecules. If the $\delta^{13}\text{C}$ values of the methyl pool in methionine was identical to that of S-adenosylmethionine, a coenzyme that derives from methionine and has $\delta^{13}\text{C}$ values of $< -39\%$ for its methyl pool (Weilacher et al., 1996), methionine could have been another source for a moderately ^{13}C -depleted methyl pool. Additionally, methionine and S-adenosylmethionine are involved in biosynthesis of many O- and S-methyl pools in organic matter, including DMSP and lignin monomers. Direct isotopic analysis of the methyl group in methionine will be essential in the future to better constrain the propagation and distribution of $\delta^{13}\text{C}$ signatures of C1 compounds in nature, including methylated sulfides.

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4.3 Implications for anoxic environments

Although our experimental temperature hinders implications for the local biogeochemistry of the lake, the incorporation of bicarbonate into DMS may be relevant to several anoxic environments, including hydrothermal fields. As summarized in Fig. 1, abiotic synthesis of alkylated sulfides has been demonstrated in the laboratory and is considered relevant for hydrothermal environments. A recent study on in situ measurements further demonstrated considerable amounts of MT in a hydrothermal area (Reeves and Seewald, 2009). The supply of MT, the usually high chemical potential of H₂ and bicarbonate, and, possibly, elevated temperature, qualify hydrothermal systems as candidate ecosystems in which the DMS-forming process characterized in this study could take place. Another relevant setting is the globally important sulfate-methane transition zone (SMTZ) in marine sediments associated with AOM activity (e.g., Hoehler et al., 2000). It is a particular zone where sulfate is exhausted and methane starts to build up. Additionally, this zone is often accompanied by peak hydrogen sulfide and bicarbonate concentrations. Laboratory experiments demonstrated that during the transition from sulfate reduction to methanogenesis, there is a decoupling of H₂ production and consumption and hence a temporary accumulation of H₂ (Hoehler et al., 1999). Isotopic evidence for acetogenesis via CO₂ reduction in an extended sediment interval just below the SMTZ at the Cascadia Margin is also consistent with elevated H₂ concentration in situ (Heuer et al., 2009). If there are sources of MT, e.g., transmethylation from lignin monomer to hydrogen sulfide, the SMTZ qualifies as an additional environment where DMS formation by methanogens is thermodynamically favorable.

5 Conclusions

In this study, we provide multiple lines of evidence for a novel microbial pathway of DMS production in anoxic lake sediment. This pathway connects DMS to bicarbonate and H₂ and is mediated by methanogens. Subsequent investigations will have to charac-

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terize the physiological conditions under which methanogens favor production of DMS and other methylated sulfides rather than methane, and to explore the environmental relevance of this novel pathway. On the other hand, our data could not identify the mechanism of MT formation despite various experimental attempts. This illustrates a more complicated biogeochemistry of MT, which will remain a great challenge for future research.

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Table 1. Concentrations of DMS and MT after 12 days of incubation with inorganic and organic substrates. Results are presented as means and standard errors from duplicate tubes.

Treatment	Headspace	DMS $\mu\text{mol (L of slurry)}^{-1}$	MT $\mu\text{mol (L of slurry)}^{-1}$
No addition	N ₂	3.7±1.7	3.0±1.7
Methanol, 800 $\mu\text{mol L}^{-1}$	N ₂	1.5±0.1	2.6±0.2
Syringic acid, 100 $\mu\text{mol L}^{-1}$	N ₂	1.1±0.4	2.7±0.1
No addition	H ₂ (+ HCO ₃ ⁻)	11.1±0.3	2.8±0.1
Sodium sulfide, 200 $\mu\text{mol L}^{-1}$	H ₂ (+ HCO ₃ ⁻)	9.6±1.0	4.4±1.6
Methanethiol, 50 $\mu\text{mol L}^{-1}$	H ₂ (+ HCO ₃ ⁻)	9.1±0.6	14.8±4.2
Methanol, 800 $\mu\text{mol L}^{-1}$	H ₂ (+ HCO ₃ ⁻)	3.7±0.4	4.3±0.1
Syringic acid, 100 $\mu\text{mol L}^{-1}$	H ₂ (+ HCO ₃ ⁻)	6.8±0.5	3.8±0.4

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Table 2. Effects of 20 mmol L⁻¹ 2-bromoethanesulfonate (BES) on formation of dimethyl sulfide, methanethiol, methane and acetate after 12 days of incubation. The sediments slurries were added with 200 kPa H₂ and 10 mmol L⁻¹ bicarbonate. Results are presented as means and standard errors from duplicate tubes.

Compounds	Positive control	+BES
Dimethyl sulfide, μmol (L of slurry) ⁻¹	11.7±0.3	4.2±2.2
Methanethiol, μmol (L of slurry) ⁻¹	3.1±0.1	4.7±0.8
Methane, μmol (L of slurry) ⁻¹	48.2±16.9	32.8±8.2
Acetate, mmol (L of slurry) ⁻¹	6.4±0.3	6.7±0.4

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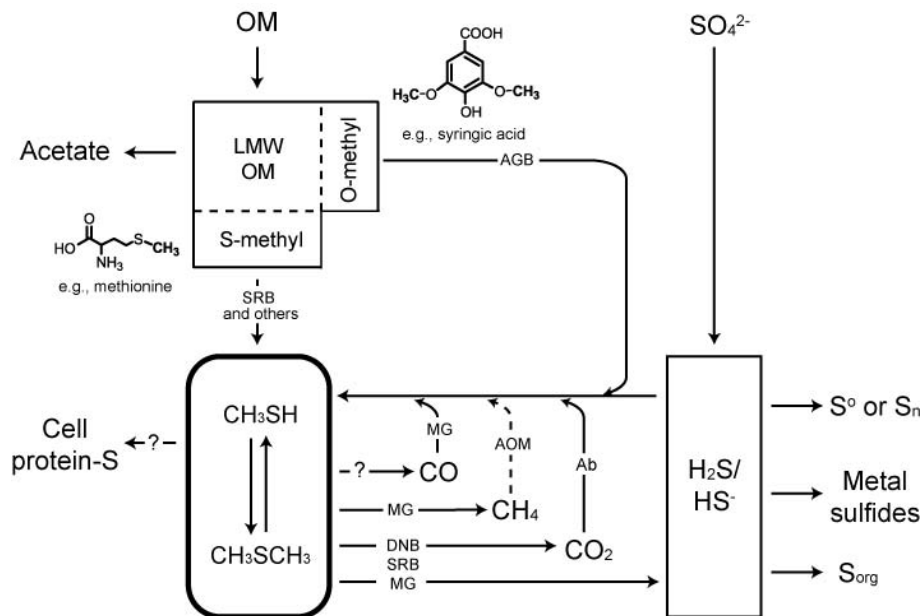


Fig. 1. A schematic diagram summarizing geochemical processes linked by methylated sulfides in anoxic environments. Arrows with dashed line: hypothesized processes; arrows with a question mark: processes that have not been examined. See text for detailed discussion. The structures of syringic acid and methionine are also shown as examples of compounds with O- and S-methyl groups, respectively. Abbreviations: Ab, abiotic process; AGB, acetogenic bacteria; AOM, anaerobic oxidation of methane; DNB, denitrifying bacteria; LMW, low molecular weight; MG, methanogens; OM, organic matter; S_{org} : sulfurized OM formed during early diagenesis; SRB, sulfate reducing bacteria.

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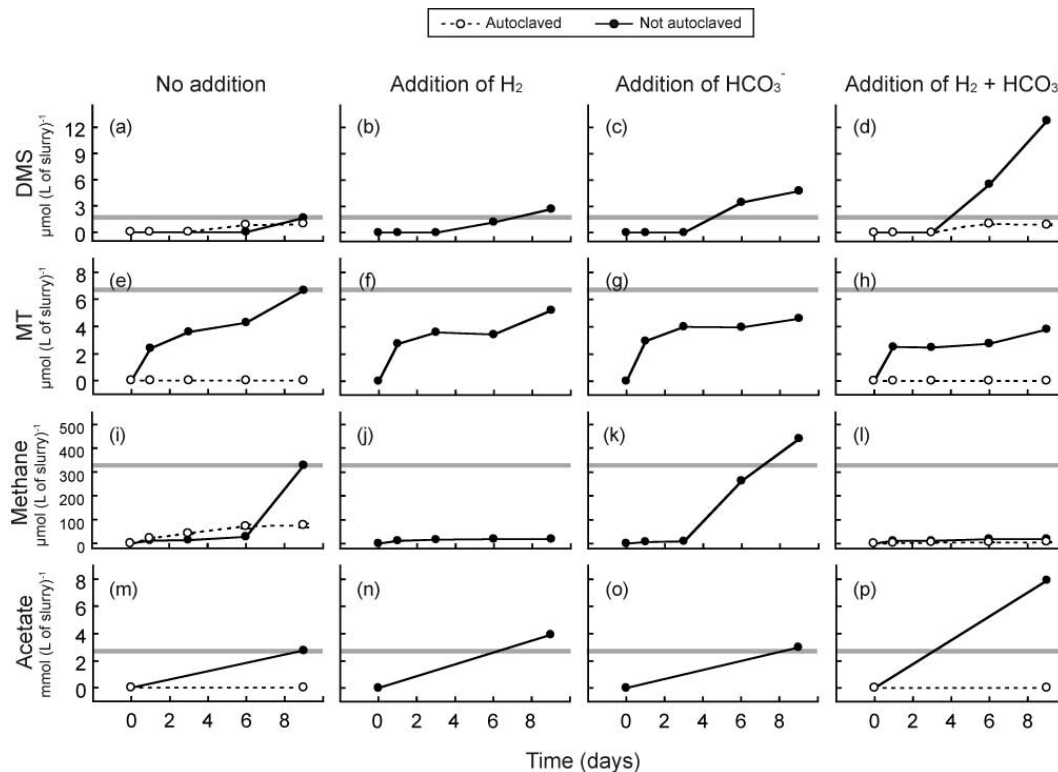


Fig. 2. Effects of H₂ and bicarbonate on the formation of dimethyl sulfide (DMS), methanethiol (MT), methane, and acetate in the sediment slurries of Lake Plußsee. Gray lines mark the final concentration of each compound in parallel with the concentration when no substrate was added. Data are from a single sample tube but the pattern is reproducible in similar incubations.

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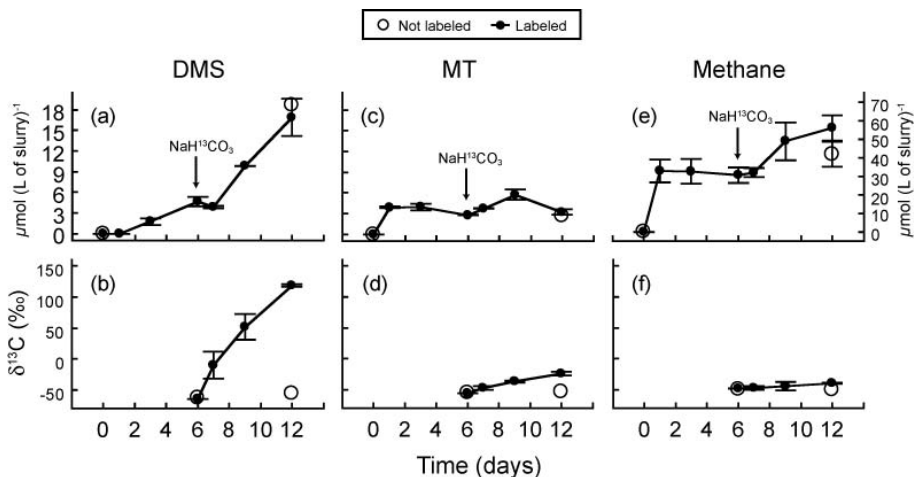


Fig. 3. Concentrations and stable carbon isotopic values of dimethyl sulfide (DMS), methanethiol (MT) and methane in sediment slurries under non-labeling and labeling conditions. $\text{NaH}^{13}\text{CO}_3$ was added on day 6 of the incubation. Error bars represent ± 1 standard error of duplicate tubes.

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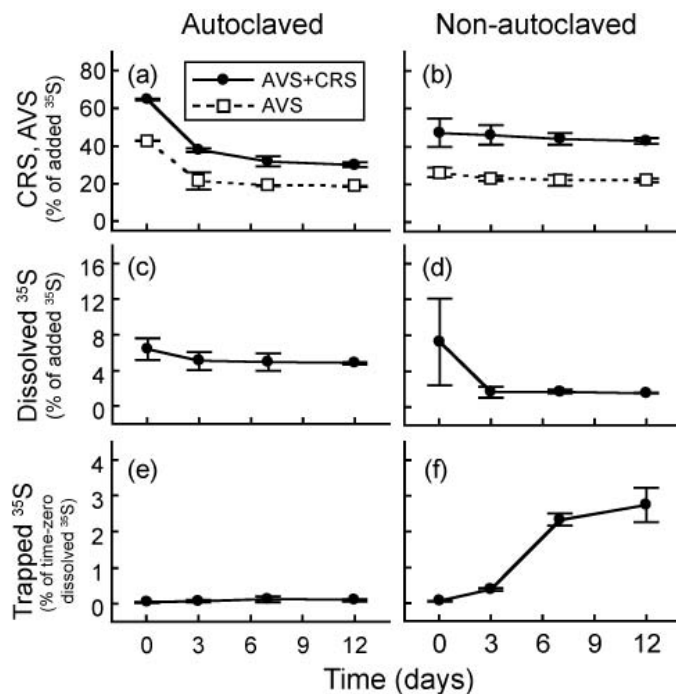


Fig. 4. Distribution of radioactivity in the sediment slurries of Lake Plußsee during incubation with H_2 , bicarbonate and $H_2^{35}S$. Time-zero values were measured two hours after addition of radiotracer. AVS: acid volatile sulfide (H_2S+FeS); CRS: chromium reducible sulfur (S^0+FeS_2). The dissolved ^{35}S was measured from filtered slurry supernatant. The trapped ^{35}S was interpreted to represent labeled dimethyl sulfide (DMS). Error bars represent ± 1 standard error of duplicate bottles.

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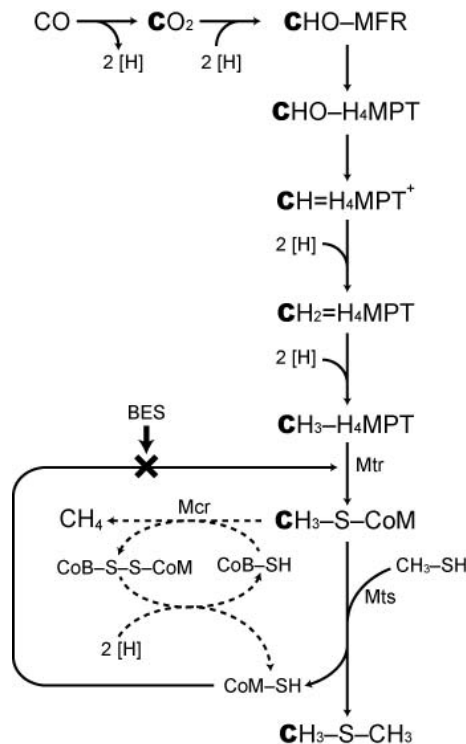


Fig. 5. A simplified scheme of methanogenic dimethyl sulfide (DMS) formation. The formation of DMS from carbon monoxide (CO) is reported in Moran et al. (2008). The carbon atoms highlighted in bold type refer to the process proposed in this study, i.e., formation of DMS from CO₂. The dashed arrows are pathways that are bypassed when DMS rather than methane is produced. The enzymatic step inhibited by 2-bromoethanesulfonic acid (BES) is marked with a cross. Abbreviations: MFR, methanofuran; H₄MPT, tetrahydromethanopterin; CoM-SH, coenzyme M; CH₃-S-CoM, methyl coenzyme M; CoB-SH, coenzyme B; Mtr, N⁵-methyl-H₄MPT:CoM-SH methyltransferase; Mcr, methyl-CoM reductase; Mts, methylthiol:CoM methyltransferase.

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